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$^{212}Pb@C_{60}$ and Its Water-Soluble Derivatives: Synthesis, Stability, and Suitability for Radioimmunotherapy

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Abstract: Fullerenes could potentially play a valuable role in radioimmunotherapy by more stably encapsulating radionuclides, especially where conventional chelation chemistry is inadequate due to the physical and/or chemical properties of the radionuclide. One of the therapeutically useful radionuclides that requires improved containment in vivo is ^{212}Pb ($\tau_{1/2} = 10.6$ h), the β-emitting parent to α-emitting ^{212}Bi ($\tau_{1/2} = 60.6$ min). Myelotoxicity resulting from the accumulation of ^{212}Pb in the bone marrow has limited the use of this radionuclide despite its favorable decay characteristics. In this work, $^{212}Pb@C_{60}$ and its malonic ester derivatives were prepared for the first time by allowing the ^{212}Pb to recoil into C_{60} following α-decay from its parent, 0.15-s ^{216}Po, generated in situ from the decay of ^{224}Ra ($\tau_{1/2} = 15$ days). Repeated washing of the organic phase containing the $^{212}Pb@C_{60}$ malonic esters with challenge solutions containing cold Pb^{2+} ions demonstrated that some of the ^{212}Pb could not be exchanged and was apparently inside of the fullerenes. Malonic esters of endohedral α-emitting ^{213}Bi ($\tau_{1/2} = 45$ min) fullerenes were prepared by an analogous procedure. Following acidification of the esters, a preliminary biodistribution study in mice was performed with the untargeted water-soluble radiofullerenes. It was found that ^{212}Pb did not accumulate in bone after being administered as an endohedral fullerene, in contrast to results with polyhydroxylated radiofullerenes and conventional polyaminocarboxylate chelators for ^{212}Pb. The results indicate that ^{212}Pb is held more tightly in the fullerene than in other methods and suggest that fullerenes may have an important role in the targeted delivery of ^{212}Pb.

Introduction

The empty interior cavity of fullerenes has long been viewed as an ideal site for containment of radionuclides during in vivo transport,[1] as one component of the effective treatment of cancer by radioimmunotherapy (RIT).[2] Since the chemistry required to open a hole in a fullerene is complex and exceedingly unlikely to occur in vivo and the conformational stability of the fullerene cage is absolute, atoms trapped within fullerenes can only be released during extremely energetic events. Encapsulating radionuclides in fullerenes has therefore been proposed as a strategy to eliminate the undesired toxicity that results from leakage and catabolism of administered radionuclides that have been complexed by other chemical methods.

In RIT, metallic radionuclides are generally contained in polyaminocarboxylate (PAC) chelators wherein three or more secondary amines and carboxylic acids chelate the radionuclide, largely sheltering it from the external environment. The radioisotopes are then delivered to cancer cells by chemically attaching the radionuclide to a chelator-modified antibody, antibody fragment, or peptide that is specific for receptors expressed preferentially on the cancer cells. Following arrival of the radionuclide−antibody conjugates at the cancer cell, energy released from the decay of the radionuclide (mainly in the form of short-range α and/or β particles as well as X-rays) can then break strands of the cell's DNA, thereby killing the cancerous cell. RIT using PAC chelators and β-emitting radionuclides has recently become an accepted method for treatment of B-cell non-Hodgkin's lymphoma (NHL), and two radiopharmaceuticals have been approved by the U.S. FDA.[3]

Effective targeting of the radionuclides remains the primary challenge in RIT. Current limitations in targeting efficacy restricts effective use of RIT to attack on cancerous cells that have preferential exposure to circulating blood (and thus the circulating antibody conjugate). The hematological malignancies represent such a tumor system that can be effectively targeted and have a significant portion of the cancerous cell volume consisting of single cells or aggregates of a few cells, typically tens of micrometers in diameter or less. In vivo, β-particles distribute their energy over millimeters from the radionuclide. Therefore, β-decays in RIT can damage adjacent healthy tissues and cells even when they are specifically targeted to cancer cells. Moreover, a single β-decay seldom breaks more than one strand of DNA, and single-strand breaks can be readily repaired.

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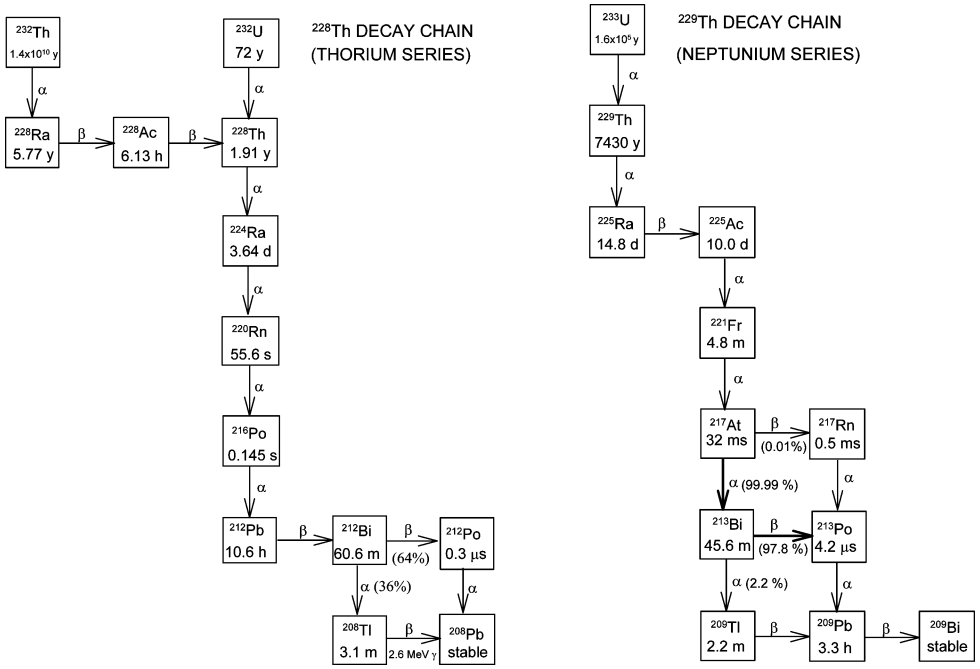

Figure 1. Decay chains referred to in the text: ^{224}Ra chain (L) and ^{225}Ac chain (R).

Microdosimetry calculations indicate that hundreds of thousands of β-decays are required to have a 99.99% chance of killing a cell.[4] Conversely, only hundreds of α-decays are required for the same probability of killing the cell, and the range of an 8 MeV α-particle (from decay of an α-emitting radionuclide) in vivo is ~100 μm or ~10 cell diameters, thereby largely limiting damage to the cells which are targeted. Although the FDA-approved NHL therapies use one of two β-emitting radionuclides (^{90}Y or ^{131}I), it is widely agreed that alpha particles have ideal characteristics for treatment of the cancers that RIT can most effectively target.[5]

Despite the favorable properties of α-particle radiation, development of α-particle RIT has been limited by the poor availability and/or physical characteristics of α-emitting radionuclides. Several reviews have debated the relative strengths and weaknesses of the available choices.[5] Currently, most effort is focused on ^{211}At, ^{225}Ac, and ^{213}Bi. The decay characteristics of ^{211}At are very attractive ($\tau_{1/2}$ = 7.2h), but using current state-of-the-art production methods,[6] about 1 day of operation at a cyclotron capable of accelerating an α-particle to a minimum energy of 28 MeV produces only about three human doses of ~8 mCi. ^{211}At is therefore most likely to be prohibitively expensive for the foreseeable future. Actinium-225 ($\tau_{1/2}$ = 10 days) is available from the decay of ^{229}Th ($\tau_{1/2}$ = 7430 yr), obtained from stockpiled ^{233}U.[7] Also, generator systems starting from ^{225}Ac have been developed for its daughter ^{213}Bi ($\tau_{1/2}$ = 45 min)[8] (Figure 1). While ^{213}Bi can be satisfactorily chelated by PAC chelators,[9] the radiochemical yield of chelation of ^{225}Ac is very small.[10] Furthermore, the α-emitting daughters of ^{225}Ac are not retained in the chelator and are released to redistribute to nontarget organs in vivo. A recent primate study with ^{225}Ac demonstrated dose-limiting renal toxicity, believed to result from decay of ^{213}Bi daughters.[10] Thus far, none of the approaches to corral the daughters have been successful,[11] and given the high recoil energy of α-decays, it is also very unlikely that even fullerenes could be of assistance in retaining the daughters of ^{225}Ac. While some encouraging results have been obtained with ^{213}Bi,[9b] its short half-life probably requires more efficient targeting than is currently available.[12]

Early RIT studies[13] using α-emitting radioisotopes were performed with ^{212}Bi ($\tau_{1/2}$ = 60.6 min), in large part because of the availability of ^{224}Ra (the parent of ^{212}Bi, see Figure 1b). The short half-life of ^{212}Bi creates the same limitations as for ^{213}Bi, but that problem can potentially be overcome using its

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parent, ^{212}Pb ($\tau_{1/2} = 10.6$ h), as a vehicle for transporting ^{212}Bi to the target tissue. However, while the PAC chelator known as DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) can chelate ^{212}Pb satisfactorily in saline solutions, in vivo tests resulted in severe myelotoxicity,[14] presumably the result of bone-targeting $^{212}Pb^{2+}$ lost or transchelated out of the DOTA. Furthermore, 36% internal conversion of the γ-ray which follows the β-decay of ^{212}Pb to ^{212}Bi also caused release of 36% of ^{212}Bi from DOTA.[15] Since we began this work, two reports of new chelators for ^{212}Pb have appeared,[16] but the results appear to indicate that further development or a new approach is still required. It was thought that use of a fullerene instead of a chelator could potentially solve both problems with ^{212}Pb, thereby making the $^{212}Pb/^{212}Bi$ system attractive for RIT. If the fullerene was successful, the only significant remaining drawback to using ^{212}Pb in RIT is the high-energy γ-ray emitted by the ^{208}Tl daughter. Compared to the outstanding problems with ^{225}Ac and ^{213}Bi, solutions for shielding health-care personnel from high-energy γ-rays are well known and relatively inexpensive compared to the costs of ^{211}At production. Thus, development of endohedral ^{212}Pb fullerenes could make α-particle RIT feasible, greatly impacting the way hematological malignancies and micrometastes are treated.

In this paper, we report the synthesis of $^{212}Pb@C_{60}$ by recoil following α-decay of its short-lived parent, formation of water-soluble $^{212}Pb@C_{60}$ malonic acids, the stability of the radiofullerene during β^--decay of ^{212}Pb to ^{212}Bi, and a preliminary biodistribution study of the untargeted water-soluble radiofullerene in mice. To our knowledge, this is the first report of an endohedral fullerene formed by recoil from α-decay, the first endohedral lead fullerene, the first fullerene encapsulating an α-emitting radionuclide with a therapeutically useful half-life, and (despite some limitations) the first biodistribution study of radiofullerene malonic acids.

Experimental Section

Radioisotopes. As needed, ^{224}Ra and ^{225}Ra were extracted from a stock of Th containing both ^{228}Th and ^{229}Th. Chemical separation of Ra and Ac from Th was achieved using the macroporous anion-exchange resin MP1 in 8 M HNO_3 media, and separation of Ra from Ac was accomplished on a low-cross-linking cation-exchange resin AG50-X4 using 1.2 M HNO_3 as eluent. A detailed procedure for these separations is given by Boll et al.[7a] At the time of separation, the activity ratio of ^{224}Ra to ^{225}Ra was 1:2.5.

Radioactivity Measurement. γ-ray spectrometry was used for measurement of all radioisotopes used in this study. The γ-ray spectrometer consisted of a calibrated intrinsic Ge detector (crystal active volume $\approx$ 100 cm^3) and a PC-based multichannel analyzer (MCA) (Canberra Industries, Meriden, CT). The detector has a resolution of 0.8 keV at 5.9 keV, 1.0 keV at 123 keV, and 1.9 keV at 1332 keV. Energy and efficiency calibrations were determined with γ-ray sources traceable to the National Institute of Standard and Technology (NIST). The γ-ray energies and their absolute intensities in parentheses used for determination of ^{212}Pb, ^{224}Ra, ^{225}Ra, and ^{213}Bi radioactivity were 238.6 (43.6%), 240.8 (3.90%), 40.1 (30.0%), and 440.4 keV (26.1%), respectively. To quantitate ^{212}Bi, the intensity of the 583.1 keV (32.5%) γ-ray of its 3.07-min ^{208}Tl daughter was measured at transient equilibrium with the parent. Consequently, at least 10 min were allowed to elapse between chemical processing and activity measurements. Since the intensity of the γ-ray from ^{225}Ac at 188.1 keV is only 0.47%, ^{225}Ac was quantitated by measurement of intensities of 218.0 keV (11.58%) from its 4.9-min ^{221}Fr daughter at transient equilibrium. All relevant nuclear data are taken from the 1986 *Table of Radioisotopes*.[17] Gross radioactivity was measured in an ionization chamber (CRC-7, Capintec Inc., NJ). An automated γ-ray scintillation counter (Wallac Wizard) consisting of a well-type NaI(Tl) detector was used for biodistribution studies of ^{212}Pb.

Synthesis by Recoil. The $^{224/225}Ra$ sources were prepared by an electrodeposition technique. The electrolysis apparatus was of conventional design consisting of a 20-mL Pyrex vial, a Pt working electrode (1.5 mm o.d. Pt rod bent into a circle and spot welded to a $\sim$1 cm diameter circle of Pt mesh), and a counter Pt electrode (1.5 mm o.d. rod). The distance between the two electrodes was $\sim$5 mm. The electrolyte, 10 mL of 0.01 M HNO_3, was purged with a gentle flow of N_2. In a typical experiment, the purified ^{224}Ra (as dry nitrate) was dissolved in 200 μL of 1.2 M HNO_3, and the mixture was transferred to the electrolysis cell. Electrodeposition was conducted under a constant potential of 12 V. Under our experimental setup, the initial current was $\sim$0.5 mA, which dropped to $\sim$0.1 mA over a 2-h period. When assay of the electrolyte indicated removal of ^{224}Ra from solution, the working electrode was carefully removed from the electrolysis cell without opening the circuit. The working electrode was then left to dry in air for 2 h and assayed for radioactivity.

A coating of C_{60} was applied by spraying a saturated toluene solution of C_{60} over the mesh with an artist's airbrush. The toluene rapidly evaporated, leaving a thick film of C_{60} over the radionuclide-plated Pt mesh. The coated mesh was then left to stand for 24−36 h, except as noted. At the end of this contact time, the coated Pt mesh was assayed for radioactivity.

Esterification. Because $M@C_{60}$ are generally insoluble,[18] the fullerenes (both C_{60} and $R@C_{60}$) were removed from the Pt mesh by derivatizing them to their malonic ester derivatives,[19] which are then soluble in tetrahydrofuran (THF) and ethyl acetate (EA). After assaying for radioactivity, the mesh was placed in a two-neck 10-mL pear-shaped flask which was purged with argon. Four milliliters of THF containing 3 mg of suspended and dispersed NaH was added. Diethylbromomalonate (0.02 mL) was also added, and the flask was vortexed vigorously for 10 min. The THF slurry was transferred by syringe from the flask into a 15-mL polypropylene conical bottom centrifuge tube and centrifuged for 2 min at $\sim$1000*g* to sediment the sodium salts. The supernatant was transferred by syringe into a fresh centrifuge tube and evaporated under argon. A 0.75 mL amount of EA was added, vortexed briefly to dissolve the fullerene esters, and then transferred to a 1.5 mL conical-bottomed centrifuge tube. Alternately, the THF could be evaporated in the flask, 0.75 mL of EA added, vortexed, removed to a 1.5 mL centrifuge tube, and centrifuged for 1 min at 10 000*g* to sediment the NaH. The EA supernatant was then transferred to a fresh 1.5 mL conical-bottomed centrifuge tube. The mesh was assayed again for radioactivity at a later convenient time.

Removal of Unencapsulated Radionuclides. Free or otherwise unencapsulated radionuclides were removed by repeated washings of the EA ester solution with an aqueous 0.012 M HNO_3 solution that was also 5 μN in each of cold Pb^{2+}, Bi^{3+}, and Tl^{3+} ions. One-half milliliter of the washing solution was added to the EA and vortexed for 10 s, followed by centrifugation at 2000 rpm for 30 s. The upper organic layer was carefully removed by a syringe with a fine-gauge

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needle and placed in a new centrifuge tube. The organic and aqueous phases were then sequentially assayed for radioactivity. The washing sequence was then repeated by contacting the EA solution with fresh aqueous acid until the aqueous wash contained less than 1 nCi of ^{212}Pb. Note that the vast majority of fullerene esters in solution after the washing are empty fullerenes.

Hydrolysis of the Esters.[19] The EA containing the ester was transferred to a 10 mL round-bottom flask and purged with N_2, evaporating the EA. A slurry of 1−2 mg of NaH in toluene was then added by syringe to the purged flask. This flask, still under N_2, was immersed in a water bath, stirred, and heated to ~80 °C for 1 h. It was then removed from the bath, and slow dropwise addition of 0.2 mL of methanol was begun immediately from a fine-gauge syringe. The reaction of the MeOH with the NaH slurry causes frothing, which could be minimized by limiting the rate of addition. Methanol addition was discontinued when a red precipitate formed and the toluene became clear. The toluene was then carefully removed with a syringe and fine-gauge needle. One milliliter of deionized water was added to the flask, which immediately dissolved the red precipitated fullerene acid. The solution was transferred to a centrifuge tube, and the solids were spun down at 10 000*g* for 5 min. All three fractions were then assayed for radioactivity.

At this point, the aqueous phase containing the ^{212}Pb@C_{60} malonic acids was extremely basic, pH ≈ 14. In the first batch, the pH was neutralized by dropwise addition of 6 M HCl. The process was improved upon in subsequent batches by adding 6 M HCl dropwise to the basic water, which fully protonates and precipitates the fullerene acid. The product was separated by centrifugation (10 000*g* for 5 min), and the acid supernatant was removed. 2-(*N*-Morpholino)ethanesulfonic acid (MES) buffer was then added to redissolve the fullerene acid. One molar NaOH was added to adjust the pH to 7. The radiofullerene acids were recovered quantitatively.

Preliminary Biodistribution Study. Female mice were obtained from Taconic, Inc., and the biodistribution studies were carried out in an AAALAC-approved animal care facility at ORNL according to approved protocols #0256 and #0267. Two different batches of ^{212}Pb@C_{60} acids were used on different dates for the in vivo testing. The first batch used ^{212}Pb@C_{60} acid that had not been reprecipitated and therefore had a high amount of salt present. To minimize the deleterious effects of the salt on the animals, two injections of radiofullerene acids were made very slowly into the tail vein, separated by 2 min. The first two mice, female severe combined immunodeficiency (SCID) mice, received 200 μL in each injection, for a total administered dose of 29.2 ± 0.1 and 28.9 ± 0.1 nCi, respectively. The third mouse was a female Balb/c mouse, and it received two 125 μL injections totaling 17.9 ± 0.8 nCi. The mice were sacrificed 1 h after injection. The following tissues were collected from each mouse and placed in previously weighed plastic scintillation vials: skin, leg muscle, femur and sternum (referred to as "bone" henceforth), stomach and intestines (which were emptied first), ovaries and uterus, liver, spleen, kidneys, heart, and lungs. Blood was also collected.

The second batch of ^{212}Pb@C_{60} acids had previously been acidified, precipitated, and redissolved as described above to minimize the extraneous salts. Six female Balb/c mice each received a single tail vein injection of 200 μL containing 10.3 ± 0.8 nCi of ^{212}Pb@C_{60} acid in pH 7 buffer. Three mice were sacrificed at 3 h postinjection and the remaining three at 8 h postinjection. The same tissues were collected as before and counted for 10 min each in the automatic γ-ray detector. Standards consisting of 10 μL of the injected solutions were also counted immediately before and after the tissues as were blank vials to provide a background. Raw data were corrected for the decay of ^{212}Pb, and % ID/g values for each sample were calculated.

Results

Electrodeposition and Adherence of Radium. Under our experimental conditions, typically 60−80% of the ^{224}Ra present electroplated within 2 h. Following the contact time with C_{60} and subsequent esterification of the fullerenes, it was found that 96.1 ± 13.2% of the electroplated ^{224}Ra remained on the platinum mesh. Conversely, no visible residue of the fullerene coating remained on the mesh after the esterification process. This result demonstrated that it is possible to reuse ^{224}Ra-electroplated meshes for many sequential esterifications.

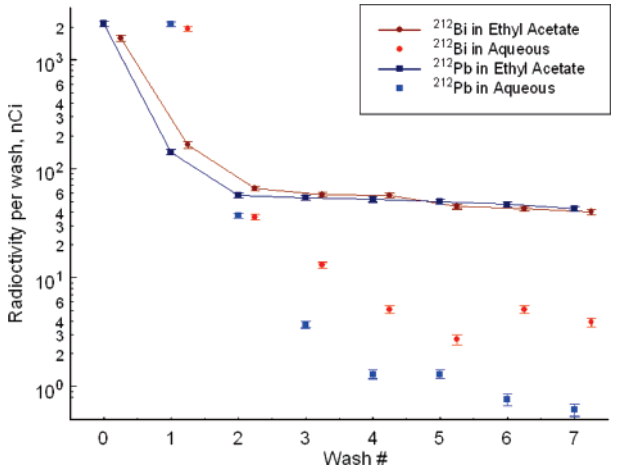

Figure 2. Radioactivity of ^{212}Pb and ^{212}Bi during aqueous washing following esterification of C_{60}. ^{212}Pb and ^{212}Bi points are offset slightly for clarity. Wash #0 is the initial radioactivity in the ethyl acetate, prior to any washing. Error bars represent the uncertainty associated with the radioactivity count rate. Logarithmic scale is used on the ordinate to show both the organic and aqueous washes on the same figure.

Behavior of the ^{224}Ra Decay Products. A typical washing sequence is shown in Figure 2 for the daughters of ^{224}Ra. The ^{212}Pb ions outside of the fullerene were expected to exchange and be extracted into the aqueous phase while the endohedral ^{212}Pb atoms remained in the ethyl acetate. For the sample shown in Figure 2, 220 nCi of ^{212}Pb@C_{60} esters (0.43% yield based on ^{212}Pb, vide infra) were recovered in the ethyl acetate after exhaustive washing. Derivatization and washing for this sample were performed over 6.5 h, although the technique was later refined to be complete in less than 1 h and could occur in about 15 min if the intermediate washes did not have to be analyzed.

Unlike ^{212}Pb, the ^{208}Tl signal in the aqueous wash stopped tailing off and attained a relatively steady value after the fourth wash (Figure 2). All of the ^{208}Tl should break out of the fullerene as it is created by α-decay from ^{212}Bi. With a half-life of 3 min, ^{208}Tl rapidly attains secular equilibrium with ^{212}Bi. After the washing was completed, the fractions were separated and after 5−10 min the ^{208}Tl signal in the ethyl acetate fraction was primarily from ^{212}Bi present in the ethyl acetate fraction, from which it decayed after the washing and before the counting. In Figure 2, 21 ± 1 min passed between washings, during which time the ^{208}Tl had attained over 99% of equilibrium to the ^{212}Bi present. By the same reasoning, the ^{208}Tl signal in the aqueous fraction indicated the presence of ^{212}Bi in the aqueous fraction, since any ^{208}Tl created before the fractions were separated would have decayed prior to counting. Thus, the values for ^{208}Tl in Figure 2 are an accurate measurement of ^{212}Bi.

To verify this, the last aqueous wash was counted at various intervals up to 2000 min after contact with the ethyl acetate (Figure 3). The observed decay for the 583 keV γ-ray could be fit with two components: an initially large component with a slope of 60.6 min (i.e., ^{212}Bi initially present in the aqueous

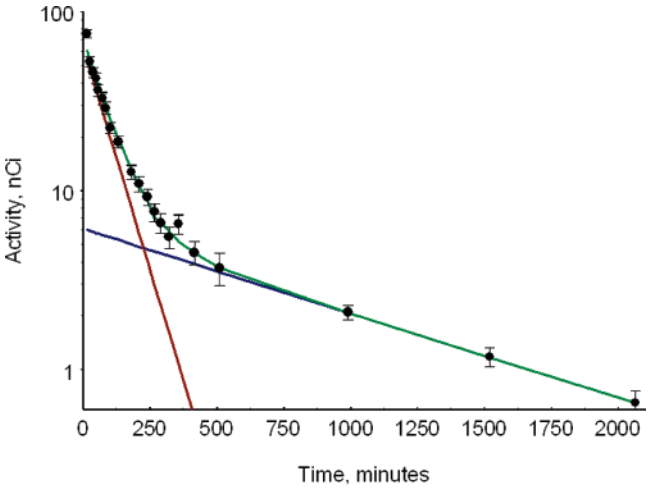

Figure 3. Counting of a single aqueous wash of R@C_{60} esters and fitting (green line) with two radionuclide decays with half-lives corresponding to ^{212}Pb (blue line) and ^{212}Bi (red line).

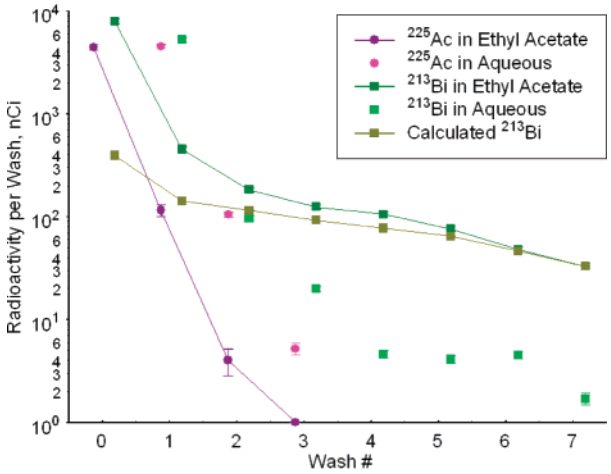

Figure 4. Activities of ^{225}Ra daughters during aqueous washing following esterification of C_{60}. Errors calculated as for Figure 1. "Calculated ^{213}Bi" is the activity of ^{213}Bi that was present in ethyl acetate at the time of that particular wash, back calculated from the activity of ^{213}Bi present at the time of Wash #7.

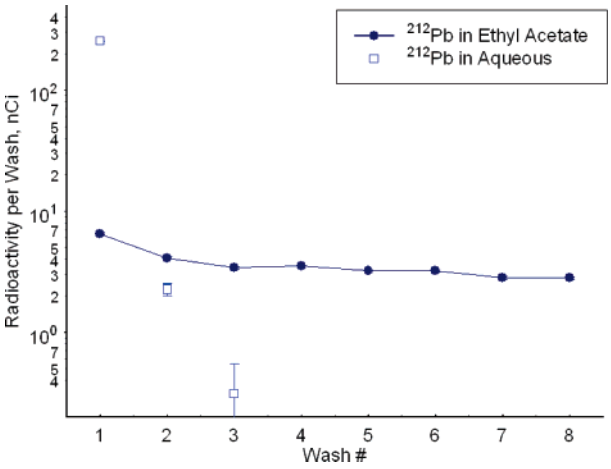

Figure 5. ^{212}Pb activity during aqueous washing following esterification of C_{60} in a sample where the C_{60} contacted the electroplated radium for only 25 min.

phase) and a very small contribution with a slope of 10.6 h (i.e., ^{212}Bi created from the decay of a minute amount of ^{212}Pb present in the aqueous, presumably from incomplete separation of the aqueous and organic layers following vortex mixing). The only possible conclusion is that a significant amount of ^{212}Bi becomes accessible to the aqueous solution during the time between each wash. The fraction of ^{212}Bi activities found in the last three aqueous washes shown in Figure 2 were 37%, 48%, and 39%, respectively, of the ^{212}Bi produced during the time elapsed since the previous wash.

Encapsulation Efficiency. The fraction of ^{212}Pb that recoiled into C_{60}, the encapsulation efficiency, was calculated from the washing data. For the sample shown in Figure 2, 150 μCi of ^{224}Ra was electroplated onto a Pt mesh. The amount of ^{212}Pb created during the 18 h of contact between the fullerene and the electroplated Ra was calculated to be 78 μCi using the well-known solution to the first-order differential equation governing the decay and growth of radioactivity (see for example ref 15), i.e., $A_2 = [(\lambda_2 - \lambda_1/\lambda_2)]*A_1{}^0[\exp(-\lambda_1 t) - \exp(-\lambda_2 t)]$, where A_2 denotes the activity of ^{212}Pb at the end of contact time, $A_1{}^0$ is the ^{224}Ra activity at the beginning of contact (150 μCi), t is the contact time (decay period), and λ_1 and λ_2 are the decay constants ($\ln 2/\tau_{1/2}$) of ^{224}Ra and ^{212}Pb, respectively. The activity of ^{212}Pb at the end of the washing period was simply back extrapolated to the end of the contact time using the relationship $A_2 = A_2{}^0[\exp(-\lambda_2 t)]$, where here $A_2{}^0$ is the ^{212}Pb activity at the end of the contact period (78 μCi), A_2 the ^{212}Pb activity after washing (220 nCi), and t the time period between the end of contact time and the end of washing (6.5 h). The decay-corrected activity of ^{212}Pb after washing was 336 nCi; therefore, the encapsulation efficiency calculated by this method was 0.336/78 = 0.43%. Using the same method, we obtained a range of values between 0.1% and 0.6% for both ^{212}Pb and ^{213}Bi, presumably varying according to the C_{60} film quality and purity of the electroplated material.

Behavior of the ^{225}Ra Decay Products. As our Ra source was a mixture of ^{224}Ra and ^{225}Ra, the decay products of ^{225}Ra could also be traced through the washing process following esterification. The distribution of ^{225}Ac (measured by its daughter ^{221}Fr) and ^{213}Bi is shown in Figure 4. No observable ^{225}Ac remained in the ethyl acetate following the second wash, while the ^{213}Bi persisted in the ethyl acetate, and after seven washes, essentially no ^{213}Bi was removed from the organic phase. Thus, ^{213}Bi behaved similarly to ^{212}Pb, while ^{225}Ac did not. Encapsulation efficiencies for ^{213}Bi were comparable to those observed for ^{212}Pb.

For one experiment, the esterification process was performed very soon after the C_{60} coating was dry, resulting in a contact time between C_{60} and the electroplated radionuclides of only 25 min, versus the more typical 18−36 h. The activities present in the washings following esterification are shown in Figure 5. The encapsulation efficiencies of ^{212}Pb and ^{213}Bi were 0.31% and 0.59%, respectively.

Conversion of Esters to Acids. Radioactivity assay of the aqueous portion after hydrolysis and separation from the toluene and the solids revealed yields between 55% and 75% for the ^{212}Pb fullerenes in the aqueous phase with ~7% of the ^{212}Pb remaining in each of the toluene and the insoluble material.

Preliminary Biodistribution Study. The results of the biodistribution study are shown in Figure 6 and Table 2. The data show that there was significant uptake in the liver and spleen. As the livers of young Balb/c mice typically weigh a little less than 1 g, around 50% of the injected dose was found

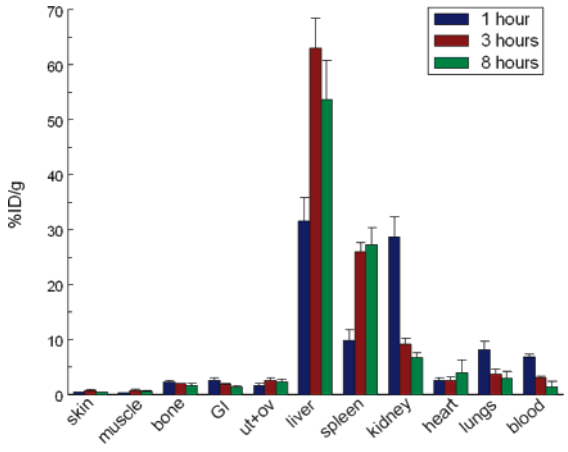

Figure 6. Average (n = 3) biodistribution of ^{212}Pb at 1, 3, and 8 h postinjection of ^{212}Pb@C_{60}(C[CO_2H]$_2$)$_x$ in female mice, showing standard deviation. GI = stomach + intenstines, ut+ov = uterus + ovaries; %ID/g = percent of injected dose per gram of tissue. See text for caveats regarding preparation of the radiofullerene, administered dose and mouse strain.

Table 1. Activities of ^{224}Ra (in μCi) on the Platinum Mesh Before and After Esterification of the Fullerene[a]

expt no.	elapsed time (h)	^{224}Ra radiaoctivity (μCi)			
		A^0	A^t(theor)	A^t(expl)	R (%)
1	18.2	51.9 ± 3.1	45.2 ± 2.7	42.5 ± 2.3	94.0 ± 7.6
2	34.0	53.8 ± 2.8	41.0 ± 2.2	41.6 ± 2.2	102 ± 8
3	53.9	33.1 ± 1.8	21.7 ± 1.2	20.0 ± 1.2	92.2 ± 7.6
				average	96.1 ± 13.2

[a] Elapsed time is the time between application of C60 on the ^{224}Ra-plated Pt mesh and dissolution of the C60 layer from Pt mesh. A^0 signifies the measured ^{224}Ra radioactivity on the Pt mesh at time zero, just before application of C60. A^t(theor) is the decay-corrected ^{224}Ra radioactivity on the Pt mesh at elapsed time t, from column 2. A^t(expl) denotes the measured ^{224}Ra radioactivity on the Pt mesh post dissolution of the C60 layer at time t (also from column 2). R is the ^{224}Ra adherence to the Pt mesh, A^t(expl) × 100/A^t(theor).

Table 2. Average (n = 3) Biodistribution of ^{212}Pb at 1, 3, and 8 h Postinjection of ^{212}Pb@C_{60}(C[CO_2H]$_2$)$_x$ in Female Mice[a]

tissue	percent injected dose per gram of tissue, % ID/g		
	1 h	3 h	8 h
liver	31.6 ± 4.3	63.1 ± 5.5	53.7 ± 7.1
spleen	9.9 ± 1.9	25.9 ± 1.7	27.2 ± 3.2
kidney	28.7 ± 3.7	9.2 ± 1.0	6.8 ± 0.9
lungs	5.2 ± 1.5	3.8 ± 0.9	2.9 ± 1.3
blood	6.9 ± 0.5	3.1 ± 0.2	1.4 ± 1.0
heart	2.5 ± 0.6	2.6 ± 0.6	4.0 ± 2.3
uterus + ovaries	1.7 ± 0.3	2.6 ± 0.5	2.3 ± 0.5
stomach + intestines	2.5 ± 0.6	1.9 ± 0.2	1.3 ± 0.3
bone	2.3 ± 0.3	2.0 ± 0.1	1.7 ± 0.3
muscle	0.4 ± 0.1	0.8 ± 0.2	0.6 ± 0.1
skin	0.5 ± 0.1	0.7 ± 0.2	0.5 ± 0.1

[a] See text for caveats regarding preparation of the radiofullerene, administered dose, and mouse strain. Values are ± the standard deviation.

in the liver between 3 and 8 h postinjection. Accumulation in the spleen was also observed, reaching about one-half the specific activity as the liver. In contrast, accumulation in bone was modest (~2% ID/g), and, most importantly, did not increase over time. None of the other tissues sampled showed high accumulation of ^{212}Pb within the limitations of the study.

Discussion

Encapsulation of Radionuclides by Recoil Following α-Particle Decay. While most Group 2 and 3 elements can be incorporated into fullerenes during the gas-phase synthesis of the fullerene, encapsulation of most other elements requires forcing the element through the fullerene cage by one means or another.[20] Thus, most of the known chemistry of endohedral fullerenes has concerned lanthanides and alkaline-earth metals, with a few reports of actinide encapsulation.[21] While there are several desirable β^--emitting radioisotopes among the lanthanides, the elements that have posed difficulties for PAC chelators are located in other parts of the periodic table. One of the more intriguing uses of fullerenes in nuclear medicine would be encapsulation of gaseous isotopes, which are unsuited to traditional methods for in vivo transport. Significant progress has been made toward that goal by neutron activating stable (gas) atoms that are mixed with fullerenes and allowing the recoil following prompt γ-ray emission to incorporate the product nuclide into the fullerene.[22] Besides lanthanides and gases, neutron activation has also been used to create endohedral fullerenes with antimony, arsenic, germanium, and selenium.[23] However, the isotopes created in these experiments do not have obvious uses in nuclear medicine. Since there are no simple neutron activation pathways that lead to α-emitting radioisotopes of interest, a new approach was required.

Part of the appeal of this method for encapsulating radioisotopes in fullerenes is that it can be performed on a very small scale without vacuum apparatus and without aerosolizing radioisotopes as would occur by the standard electric arc or laser ablation processes for generating endohedral metallofullerenes. It was not a priori apparent that endohedral metallofullerenes could be formed from recoil of the daughter nucleus following α-decay. Recoil energies associated with α-decay are much higher than those from prompt γ-ray emission following neutron capture. Rare-earth and noble-gas nuclides have kinetic energies between 100 and 500 eV due to the recoil imparted by the emission of a γ-ray following neutron capture. The kinetic energy of the newly formed ^{212}Pb nucleus (after decay of ^{216}Po) is ~33 keV, almost 2 orders of magnitude larger than the recoil energy of any previously implanted nuclide. Conversely, β^--decays typically impart 10−20 eV of recoil energy to the daughter nucleus (except for very low Z nuclei) and are generally too small to implant or remove a nucleus from C_{60} (ref 15). However, molecular dynamics simulations show that a nucleus with as little as 5 eV kinetic energy can penetrate the fullerene wall in the extremely low probability case that it is aimed exactly at the middle of a hexagon in the fullerene.[23] Thrash et al. convincingly established that recoil energies less than 200 eV are sufficient to remove a ^{166}Ho nucleus from the inside of C_{60}.[24] Due to the rapid recoil of the nucleus relative to the kinetic energy of its electrons, some outer electrons will be stripped off of the ^{212}Pb radioisotope at the time of its creation. As the

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(21) (a) Guo, T.; Diener, M. D.; Chai, Y.; Alford, J. M.; Haufler, R. E.; McClure, S. M.; Ohno, T.; Weaver, J. H.; Scuseria, G. E.; Smalley, R. E. *Science* **1992**, *257*, 1661−3. (b) Akiyama, K.; Zhao, Y.; Sueki, K.; Tsukada, K.; Haba, H.; Nagame, Y; Kodama, T.; Suzuki, S.; Ohtsuki, T.; Sakaguchi, M.; Kikuchi, K.; Katada, M.; Nakahara, H. *J. Am. Chem. Soc.* **2001**, *123*, 181−2. (c) Diener, M. D.; Smith, C. A.; Veirs, D. K. *Chem. Mater*. **1997**, *9*, 1773.
(22) (a) Gadd, G. E.; Evans, P. J.; Hurwood, D. J.; Wood, J.; Morica, S.; Blackford, M.; Elcombe, M.; Kennedy, S.; James, M. *Chem. Phys. Lett.* **1996**, *261*, 221−7. (b) Jimenez-Vasquez, H. A.; Cross, R. J.; Saunders, R.; Poreda, R. J. *Chem. Phys. Lett.* **1994**, *229*, 111−4.
(23) Ohtsuki, T.; Ohno, K.; Shiga, K.; Kawazoe, Y.; Maruyama, Y.; Masumoto, K. *J. Chem. Phys.* **2000**, *112*, 2834.
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velocity of the recoiling ^{212}Pb decreases, it will regain electrons from its surroundings. Theoretically, the range of $^{212}Pb^{2+}$ ions with kinetic energy of 32 keV in graphite is ~2 μm, corresponding to roughly 1500 C_{60} molecules. Therefore, the ^{212}Pb ions likely smash through over a thousand fullerenes before losing enough energy to stay encapsulated. This may account for the relatively low encapsulation efficiency.

It was found that little of the parent radioisotope ^{224}Ra was removed from the source mesh during removal of the fullerenes by esterification (Table 1). Potentially, many films of fullerenes could therefore be applied and removed from a single source of electroplated ^{224}Ra. Removal of the esterified $^{212}Pb@C_{60}$ daughters from the ^{224}Ra electroplated and immobilized on the mesh is akin to a conventional radioisotope generator in that the daughters can be extracted every so often without disturbing the parent radionuclides. Even with 1% encapsulation efficiency, four human doses (estimated as 5 mCi) of $^{212}Pb@C_{60}$ could be produced from 1 Ci of ^{224}Ra in a 5-day week. Theoretically, 3.8 Ci of ^{224}Ra can be extracted every week from a 5 Ci stockpile of ^{228}Th. With a half-life of slightly under 2 years, 5 Ci of ^{228}Th can produce roughly 750 doses of $^{212}Pb@C_{60}$ over ~3 years, which would be suitable for a Phase II clinical trial. Furthermore, irradiating 1 g of ^{226}Ra in the ORNL High Flux Isotope Reactor can produce ~400 Ci of ^{228}Th[7c]—sufficient for production of 60 000 doses of $^{212}Pb@C_{60}$ over a 3-year period. An encapsulation efficiency of 1% is therefore not prohibitive to a therapy based on $^{212}Pb@C_{60}$.

Radiolytic Purification of $^{212}Pb@C_{60}$ Malonic Esters. The principle behind washing the esterified solutions is that radioactive metal ions outside of the fullerene exchange rapidly with the cold metal ions present in the aqueous washing solution. The exchange occurs because there are several orders of magnitude more cold metal ions than radioactive ones. Although the enthalpies of the exchange reactions are zero, the free energies are negative due to the increase in entropy of the systems. However, if the radioactive metal ion is inside of a fullerene ester (which is not soluble in water), it cannot exchange through the fullerene cage and remains in the organic phase. The esters can be dissolved in toluene (instead of ethyl acetate) and then washed, but it requires more washes to remove the exohedral radionuclides from toluene solution than when ethyl acetate is used as the organic solvent.

Note that this procedure will readily exchange radioactive metal atoms from PAC chelators as well as other chemically bonded systems. Failure to exchange the ^{212}Pb out of the ethyl acetate solution provides definitive evidence that the ^{212}Pb is located inside of the fullerene. Furthermore, the encapsulated ^{212}Pb is exceedingly unlikely to be transchelated in vivo as there is no evidence to suggest that a hole can be opened in the fullerene by any enzymatic action in a time frame comparable the 10.6-h half-life of ^{212}Pb.

This theory was corroborated by the finding that ^{225}Ac was not retained in the organic phase. Since ^{225}Ac is produced by β^--decay from ^{225}Ra, it does not have enough recoil energy to penetrate a fullerene wall. On the other hand, daughters by α-decay of both the ^{224}Ra and the ^{225}Ra decay chains are found in the C_{60} with similar encapsulation efficiencies. Reducing the contact time between the fullerenes and the radionuclides greatly reduced the amount of radiofullerenes produced, but the encapsulation efficiencies remained essentially constant. Since the chemical procedures carried out were identical regardless of contact time, hypotheses regarding some chemical derivatization of Pb and Bi (but not Ac) to impart organic solubility can be ruled out. Additionally, such hypotheses would require derivatization to occur in some unknown way where the radionuclide is not subject to exchange during washing.

Fate of ^{212}Bi Following β—Decay of ^{212}Pb. Previous work showed that ~36% of the ^{212}Bi formed from a DOTA-chelated ^{212}Pb was lost from the chelator.[15] This is significant because 36% of the time the 238-keV γ-ray which follows β^--decay of ^{212}Pb undergoes internal conversion (IC).[15] In the IC process, the electromagnetic interaction between the excited nucleus and the electronic shell results in ejection of electrons (mainly from the K shell). The ejected electrons would have a kinetic energy equal to the nucleus excitation energy minus the electron binding energy. From the point of view of an external observer, however, the process appears as an internal photoelectric effect, i.e., a γ-ray collides with an electron, ejecting the electron out of its orbit. For an atom, the consequence of an IC process is similar to that of a K-capture (KC) process. The positive charge generated in the sea of electrons is immediately filled by the electrons from upper shells, generating corresponding X-rays which, in turn, may under go internal photoelectric effect, thereby knocking out electrons from upper shells. The cascade of the event through the electronic shells always results in loss of a number of electrons from the binding shells, leaving the atoms highly charged. If a radioactive atom undergoing an IC or KC process is part of a molecule, then the daughter atom is almost always ejected from the molecule. This is due to the fact that the electron transfers within the molecule, needed to quench the excitation generated by IC or KC, are very slow relative to nuclear events.

The net result is that in the 36% of the time where IC occurs, ^{212}Bi is created in a highly ionized state (+5 or even higher). Experimentally, the portion of the ^{212}Bi created during the time between washings that was extracted into the aqueous phase on successive washes (39%, 48%, and 37%) correlates reasonably well with the percent of ^{212}Bi that undergoes internal conversion (36%). It therefore seems most likely that the internal conversion process ejected ^{212}Bi ions from the fullerene ester or otherwise modified the fullerene ester so as to make it water soluble.

While it was originally anticipated that the fullerene would retain all of the ^{212}Bi after β^--decay from ^{212}Pb, the loss of 36% of ^{212}Bi should be put in perspective relative to ^{225}Ac-based strategies. Consider that all of the ^{221}Fr created by β-decay from ^{225}Ac (as well as its two daughters) will be separated from any chelator (or fullerene) and left to deposit their energy according to the biochemistry of the daughter ions. In the ^{225}Ac case, ca. 300% of the administered dose is lost, compared to 36% loss for endohedral ^{212}Pb.

Biodistribution of the Untargeted $^{212}Pb@C_{60}$ Malonic Acids. The preliminary biodistribution results showed slow clearance of $^{212}Pb@C_{60}$ malonic acids relative to PAC chelators, which are largely expelled within an hour. This was also the case with the only other biodistribution study of radiofullerenes.[25] That work studied the biodistribution of $^{166}Ho_x@C_{82}(OH)_y$ ($x = 1,2$; $y = 16-24$), and found 20−25%

(25) Cagle, D. W.; Kennel, S. J.; Mirzadeh, S.; Alford, J. M.; Wilson, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5182.

ID/g in the liver between 1 and 4 h, with it clearing slowly afterward. We hoped that derivatization by malonic acids would improve the clearance of the radiofullerene relative to polyhydroxylate derivatives, but that is clearly not the case. However, slow clearance of untargeted radiofullerenes should be irrelevant following proper conjugation to an antibody or other targeting agent.

One crucial aspect of the biodistribution which did greatly improve was the accumulation in bone. Over 8% ID/g of the polyhydroxylate was present in the bone at 1 h, and that figure increased to over 11% ID/g by 24 h.[25] Conversely, the malonate investigated herein accumulated only ~2% ID/g in the bone. It is possible that some $^{212}Pb@C_{60}$ malonate localizes in the bone due to formation of ionic and hydrogen bonds between mineralized bone and the partially protonated form of the malonic acid groups used to induce water solubility in the radiofullerene.[26] If this were true, it would explain why 2% ID/g was found in the bone when none was expected and imply that a different water-solubilizing group could potentially further reduce radiofullerene accumulation in bone. In any case, the observed ~2% ID/g in the bone is comparable to the best value obtained from a new set of PAC chelators.[16b] In that work, the % ID/g found in the femur for ^{203}Pb was much higher than for the other radionuclides tested in the PAC chelators (^{177}Lu, ^{86}Y, and $^{205/6}Bi$), indicating the continuing need for improved methods for in vivo transport of lead and other bone-seeking radioisotopes.

In contrast to chelation, the fullerene should be able to maintain containment of ^{212}Pb under any in vivo condition, as the ability of the fullerene to retain encapsulation of an atom is unaffected by pH, intermolecular complexation, or charge state of the internal atom. The presence of any lead in the bone after encapsulation within a fullerene is therefore surprising. Initially, perhaps some activity could be attributed to blood and/or adventitial tissues. Given the generally slow clearance of the fullerene malonates, perhaps this is still the case, even after 8 h. A longer biodistribution study, with higher administered activities, will be required to determine the cause of the observed ^{212}Pb activity in the bone as well as establish the clearance rate of the fullerene malonic acid. Application to RIT will ultimately require conjugation of the fullerene malonates to antibodies (or other targeting agents), which has recently been accomplished for the first time.[27]

(26) As pointed out by a reviewer: Wilson, T. M.; Charifson, P. S.; Baxter, A. D.; Geddie, N. G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1043.
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Conclusions

For the first time, endohedral metallofullerenes were prepared containing α-emitting radionuclides suitable for use in radioimmunotherapy, specifically ^{212}Pb and ^{213}Bi. A new preparation route for radiofullerenes, using the recoil following α-decay of a parent radionuclide, was also demonstrated. Malonic esters were then added to the radiofullerenes, and repeated washing of ethyl acetate solutions of the radiofullerene esters with aqueous challenge solutions was found to be a rapid technique for removal of exohedral radionuclides. While $^{212}Pb@C_{60}$ appeared stable in solution, about 36% of the ^{212}Bi formed within the C_{60} by β-decay from the $^{212}Pb@C_{60}$ was apparently released from the fullerene or otherwise damaged the fullerene. In a preliminary biodistribution study, encapsulation of ^{212}Pb in C_{60} malonic acid derivatives prevented the ^{212}Pb from accumulating in bone but demonstrated rather slow clearance. This work demonstrates the potential of fullerenes for solving problems that have not been solved by PAC chelators but also illustrates some limitations to the use of fullerenes.

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